Role of Activin, Follistatin, and Inhibin in the Regulation of Kiss-1 Gene Expression in Hypothalamic Cell Models

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Abstract

Kisspeptin (encoded by the Kiss-1 gene) in the arcuate nucleus (ARC) of the hypothalamus governs the hypothalamic-pituitary-gonadal (HPG) axis by regulating pulsatile release of gonadotropin-releasing hormone (GnRH). Meanwhile, kisspeptin in the anteroventral periventricular nucleus (AVPV) region has been implicated in estradiol (E2)-induced GnRH surges. Kiss-1–expressing cell model mHypoA-55 exhibits characteristics of Kiss-1 neurons in the ARC region. On the other hand, Kiss-1 expressing mHypoA-50 cells originate from the AVPV region. In the mHypoA-55 ARC cells, activin significantly increased Kiss-1 gene expression. Follistatin alone reduced Kiss-1 expression within these cells. Interestingly, activin-induced Kiss-1 gene expression was completely abolished by follistatin. Inhibin A, but not inhibin B reduced Kiss-1 expression. Activin-increased Kiss-1 expression was also abolished by inhibin A. Pretreatment of the cells with follistatin or inhibin A significantly inhibited kisspeptin- or GnRH-induced Kiss-1 gene expression in mHypoA-55 cells. In contrast, in the mHypoA-50 AVPV cell model, activin, follistatin, and inhibin A did not modulate Kiss-1 gene expression. The subunits that compose activin and inhibin, as well as follistatin were expressed in both mHypoA-55 and mHypoA-50 cells. Expression of inhibin βA and βB subunits and follistatin was much higher in mHypoA-55 ARC cells. Furthermore, we found that expression of the inhibin α subunit and follistatin genes was modulated in the presence of E2 in mHypoA-55 ARC cells. The results of this study suggest that activin, follistatin, and inhibin A within the ARC region participate in the regulation of the HPG axis under the influence of E2.

Introduction

The hypothalamic-pituitary-gonadal (HPG) axis regulates the modulation and maintenance of reproductive functions. In mammals, early studies on the regulation of the HPG axis have emphasized the pivotal role of gonadotropin-releasing hormone (GnRH) neurons located in the preoptic area (POA) of the hypothalamus [1]. GnRH is delivered via portal circulation and induces the secretion of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which in turn control gametogenesis and sex steroid synthesis in the gonads. After the discovery of the loss of function of the gene encoding the receptor for the hypothalamic peptide kisspeptin (Kiss1R) [2, 3], our understanding of the neuronal mechanisms controlling the HPG axis was greatly advanced and now it is generally agreed that kisspeptin, which is produced in hypothalamic Kiss-1 (the gene that encodes kisspeptin) neurons, is positioned at the highest level in the HPG axis and controls the release of GnRH from neurons [4].

In rodents, Kiss-1 neurons are mainly located in two different areas of the hypothalamus, the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) [5, 6], but Kiss-1 is also expressed in the amygdala [7]. Kiss-1 neurons in both the AVPV and ARC project to the POA where GnRH neurons are located [8]. Most of the GnRH neurons express Kiss1R and are activated and secrete GnRH upon kisspeptin stimulation [9, 10]. Kiss-1 neurons in these two areas are implicated in the estradiol-induced (E2-induced) positive and negative feedback mechanisms based on the observations that Kiss-1 expression in the AVPV nucleus is upregulated by E2, whereas Kiss-1 expression within the ARC nucleus is repressed by E2 [5, 11, 12].

The HPG axis also consists of activin, inhibin, and follistatin produced by the gonads. Activin has been identified as a gonadal peptide that stimulates FSH secretion

from the pituitary [13]. Structurally, activins are homo- and heterodimeric proteins containing two disulphide-linked βA (encoded by the *Inhba* gene) and/or βB (encoded by the *Inhbb* gene) subunits [13]. Among activin A (βA/βA), activin B (βB/βB), and activin AB (βA/βB), activin A and B are known to be associated with reproduction [14]. In contrast, inhibin and follistatin have an antagonistic effect on activin, and reduce FSH synthesis and secretion [15, 16]. Inhibins secreted from ovarian granulosa cells have a structure similar to that of activins, and are heterodimeric proteins consisting of an α subunit (inhibin α) and βA (inhibin A) or βB (inhibin B) subunit [17]. Inhibin competitively binds the activin receptor, thus supressing activin activity [18]. Follistatin was first discovered in porcine ovarian follicular fluid and inhibits the release of FSH [19]. Follistatin has a different structure from that of activin and inhibin. It can bind directly and tightly with activin and hinder activin's binding to its receptor, and it can also accelerate the internalization and degradation of activin [20]. Activin, inhibin, and follistatin are produced in the ovary and synthesis of these peptides changes according to the reproductive phase in humans [21, 22]. Furthermore, these ovarian peptides also modulate pituitary gonadotropin synthesis and secretion, suggesting that these peptides also participate in the control of the HPG axis.

Expression of activin, inhibin, and follistatin subunits and proteins has been detected in the central nervous system including the hypothalamus in humans [23, 24]. In this study, we used two hypothalamic Kiss-1–expressing cell models and examined the effects of activin, inhibin, and follistatin. mHypoA-55 cells are a model for mouse Kiss-1–expressing neurons in the ARC region of the hypothalamus. This cell model was established from microdissected ARC primary cultures of female adult mouse hypothalamus and exhibit Kiss-1 as well as estrogen receptor α (ERα) and

ERβ expression. In addition, they express neurokinin B (NKB) and dynorphin (Dyn), suggesting that they represent kisspeptin-NKB-Dyn (KNDy) neurons in the ARC region of the hypothalamus. On the other hand, Kiss-1–expressing cells that originated from the AVPV region of mouse hypothalamus were named mHypoA-50 cells, and these cells are devoid of NKB and Dyn expression [25]. Interestingly, these cells exhibit a different response to E2 in the regulation of the Kiss-1 gene. E2 downregulated Kiss-1 expression in nHypoA-55 cells under certain experimental conditions, but it increased Kiss-1 expression in mHypoA-50 cells. Therefore, these two cells are used as models for the study of the mechanisms of E2-induced negative and positive feedback control [25]. Using these hypothalamic Kiss-1–expressing cell models, we examined possible roles of the gonadal peptides activin, inhibin, and follistatin in Kiss-1 neurons.

Materials and Methods

Materials

The following chemicals and reagents were obtained from the indicated sources: GIBCO fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA); Dulbecco's modified Eagle's medium (DMEM), GnRH, follistatin, water-soluble E2, and penicillin-streptomycin (Sigma-Aldrich Co., St. Louis, MO); activin A and inhibin B (Abcam, Cambridge, MA); inhibin A (R&D Systems, Inc., Minneapolis, MN); and kisspeptin-10 (ANA SPEC, Fremont, CA).

Cell culture and stimulation

mHypoA-55 and mHypoA-50 cells were purchased from CEDARLANE (Ontario, Canada). Cells were plated in 35-mm tissue culture dishes and incubated with high-glucose DMEM containing 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37°C under a humidified atmosphere of 5% CO_2 in air. After 24 h, cells were used for each experiment. While stimulated with the test reagents, cells were incubated without (control) or with the test reagents in high-glucose DMEM containing 1% heat-inactivated FBS and 1% penicillin-streptomycin for the indicated concentrations and time periods. While stimulated by E2, cells were cultured with sex steroids in phenol red–free DMEM supplemented with 1% charcoal-stripped FBS (Gemini Bio-Products, West Sacramento, CA).

RNA preparation, reverse transcription, PCR, and quantitative real-time PCR

Total RNA from stimulated cells was extracted using TRIzol-LS (Invitrogen) according to the manufacturer's instructions. To obtain cDNA, 1.0 μg total RNA was

reverse transcribed using an oligo-dT primer (Promega, Madison, WI) and prepared using a First-Strand cDNA Synthesis Kit (Invitrogen) in reverse transcription (RT) buffer. The preparation was supplemented with 10 mM dithiothreitol, 1 mM of each dNTP, and 200 U RNase inhibitor/human placenta ribonuclease inhibitor (Code No. 2310; Takara, Tokyo, Japan) in a final volume of 10 µl. The reaction was incubated at 37°C for 60 min. For the detection of inhibin α, inhibin βA, and inhibin βB subunit mRNAs, after PCR amplification using primers for inhibin α (forward: 5′-GTGGGGAGGTCCTAGACAGA-3′ and reverse: 5′-GTGGGGATGGCCGGAATACA-3′), inhibin βA (forward: 5′-GGAGTGGATGGCAAGGTCAACA-3′ and reverse: 5′-GTGGGCACACAGCATGACTTA-3′), inhibin βB (forward: 5′-GGTCCGCCTGTACTTCTTCGTCT-3′ and reverse: 5′-GGTATGCCAGCCGCTACGTT-3′), and follistatin (5′-GTGACAATGCCACATACGCC-3′ and reverse: 5′-GCCTCTGCAGTTACGCAATAA-3′), amplicons were electrophoresed in agarose gels and visualized with ethidium bromide staining. cDNAs from rat cerebral cortex, rat ovary, rat hypothalamus, and rat anterior pituitary were used as positive controls. Quantification of Kiss-1, inhibin α subunit, and follistatin mRNAs were obtained through quantitative real-time PCR (ABI Prism 7000; Perkin-Elmer Applied Biosystems, Foster City, CA) following the manufacturer's protocol (User Bulletin No. 2) and utilizing Universal ProbeLibrary Probes and FastStart Master Mix (Roche Diagnostics, Mannheim, Germany). Using specific primers for mouse Kiss-1 (forward: 5′-ATGATCTCGCTGGCTTCTTGG-3′; reverse: 5′-GGTTCACCACAGGTGCCATTTT-3′), inhibin α, and follistatin, the simultaneous measurement of mRNA and GAPDH permitted normalization of the amount of cDNA added per sample. For each set of primers, a no-template control was included. Thermal

cycling conditions were as follows: 10 min denaturation at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Reactions were followed by melting curve analysis (55°C–95°C). To determine PCR efficiency, a 10-fold serial dilution of cDNA was performed as previously described [26]. PCR conditions were optimized to generate >95% PCR efficiency and only those reactions with between 95% and 105% efficiency were included in subsequent analyses. Relative differences in cDNA concentration between baseline and experimental conditions were then calculated using the comparative threshold cycle (Ct) method [27]. Briefly, for each sample, a ΔCt was calculated to normalize to the internal control using the following equation: ΔCt = ΔCt(gene) – Ct (GAPDH). To obtain differences between experimental and control conditions, ΔΔCt was calculated as ΔCt(sample) – ΔCt(control). Relative mRMA levels were then calculated using the following equation: fold difference = $2^{\Delta\Delta Ct}$.

Western blot analysis

Cell extracts were lysed on ice with RIPA buffer (phosphate-buffered saline [PBS], 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) containing 0.1 mg/ml phenylmethyl sulfonyl fluoride, 30 mg/ml aprotinin, and 1 mM sodium orthovanadate, scraped for 20 s, and centrifuged at 14,000 × *g* for 10 min at 4°C. Protein concentration in the cell lysates was measured using the Bradford method. Denatured protein (10 μg per well) was resolved in a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel according to standard protocols. Protein was transferred onto polyvinylidene difluoride membranes (Hybond-P PVDF, Amersham Biosciences, Little Chalfont, UK), which were blocked for 2 h at room temperature in Blotto (5% milk in Tris-buffered saline). Membranes were incubated with

anti-kisspeptin antibody (1:100 dilution: Abcam), anti-inhibin α antibody (1:100 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX), anti-inhibin βA antibody (1:100 dilution; Santa Cruz Biotechnology, Inc.), anti-βB antibody (1:1000 dilution; Abcam), or anti-follistatin (1:100 dilution; Santa Cruz Biotechnology, Inc.) in Blotto overnight at 4°C and washed 3 times for 10 min per wash with Tris-buffered saline/1% Tween. Subsequent incubation with horseradish peroxidase–conjugated (HRP-conjugated) antibodies was performed for 1 h at room temperature in Blotto, and additional washes were performed as needed. Following enhanced chemiluminescence detection (Amersham Biosciences), membranes were exposed to X-ray film (Fujifilm, Tokyo, Japan). Tissues from rat cerebral cortex, rat ovary, and rat anterior pituitary were used as positive controls. This experimental protocol was approved by the animal care and use committee of the Experimental Animal Center for Integrated Research at Shimane University (IZ27-82). To compare the expression levels of inhibin α, βA, βb, and follistatin in mHypoA-50 and mHypoA-55 cells, films were analyzed by densitometry, and the intensities of these protein bands were normalized to those of β-actin to correct for protein loading using an anti-β-actin primary antibody (1:100 dilution; Abcam) and HRP-conjugated secondary antibody.

Statistical analysis

All experiments were repeated independently at least three times. Each experiment in each experimental group was performed using duplicate samples. When we determined the mRNA expression, two samples were assayed in duplicate. Six averages from three independent experiments were statistically analyzed. Data are expressed as mean ± standard error of the mean (SEM) values. Statistical analysis was

performed using one-way analysis of variance (ANOVA) with Bonferroni's post hoc test, or Student's *t* test, as appropriate. $P < 0.05$ was considered statistically significant.

Results

Effect of activin on Kiss-1 gene expression in the mHypoA-55 ARC cell model

First, we examined the effect of activin on Kiss-1 gene expression in the ARC cell model mHypoA-55. Activin stimulation significantly increased Kiss-1 mRNA expression in mHypoA-55 cells by 3.424 ± 0.75-fold at a concentration of 1 ng/ml and 3.15 ± 0.36-fold at 10 ng/ml (Fig. 1A). Time-course experiments demonstrated that activin (10 ng/ml) increased Kiss-1 gene expression at 24 h after stimulation, but failed to increase its expression at earlier time points (Fig. 1B). Activin's effect on Kiss-1 gene expression was also observed at 48 h after stimulation, but the increase was not statistically significant between 24 and 48 h after activin stimulation (data not shown). Western blotting analysis using a specific anti-kisspeptin antibody showed that kisspeptin protein expression within mHypoA-55 cells was dramatically increased by treatment with activin (Fig. 1C).

Effect of follistatin on Kiss-1 gene expression in mHypoA-55 cells

Next, we examined the effect of follistatin on Kiss-1 gene expression in mHypoA-55 ARC cells. Follistatin stimulation for 24 h significantly inhibited basal expression of Kiss-1 mRNA in mHypoA-55 cells. With 10 ng/ml follistatin stimulation, Kiss-1 mRNA expression was reduced by almost 40% compared with non-treated cells (Fig. 2A). Time-course experiments showed that the follistatin-mediated decrease in Kiss-1 gene expression occurred relatively early. Follistatin (10 ng/ml) reduced basal Kiss-1 gene expression by nearly 60% at 6 h after stimulation, and it was still reduced at 24 h compared with non-treated cells (Fig. 2B). Although follistatin significantly reduced Kiss-1 gene expression until 24 h after stimulation, a significant reduction was not

observed at 48 h after stimulation (data not shown). We also confirmed that the kisspeptin protein level in mHypoA-55 cells was reduced by the treatment with follistatin (Fig. 2C).

Effect of follistatin on activin-induced Kiss-1 gene expression in mHypoA-55 cells

Activin stimulation increased Kiss-1 mRNA expression in mHypoA-55 cells, as described above. Combined treatment with activin and follistatin completely abolished activin's effect on Kiss-1 mRNA expression; indeed, follistatin reduced the Kiss-1 expression to below that of the control level even when activin was present. Activin significantly increased Kiss-1 gene expression up to 1.35 ± 0.081-fold in this experiment, but in the presence of follistatin it was significantly reduced to 0.58 ± 0.06-fold compared with non-treated cells (Fig. 3).

Effect of inhibin on the expression of Kiss-1 in mHypoA-55 cells

Next, we examined the effect of inhibin on Kiss-1 expression in mHypoA-55 ARC cells. In humans, inhibin B is increased during the late follicular phase, whereas inhibin A is elevated during the luteal phase [28]. Kiss-1 mRNA expression in mHypoA-55 cells was reduced by inhibin A treatment. Stimulating the cells with inhibin A significantly reduced basal expression of Kiss-1 mRNA, which was reduced to 0.69 ± 0.16-fold at 1 ng/ml and to 0.59 ± 0.13-fold at 10 ng/ml compared with non-treated cells (Fig. 4A). In contrast, neither 1 ng/ml nor 10 ng/ml inhibin B modulated the expression of Kiss-1 mRNA in mHypoA-55 cells (Fig. 4B). Inhibition of Kiss-1 gene expression by inhibin A was observed relatively quickly (6 h after treatment), and its inhibitory effect remained until 24 h after inhibin A treatment (Fig. 4C). At 48 h after inhibin A treatment, Kiss-1 gene expression was not significantly different compared with the non-stimulated

controls (data not shown). We thus confirmed that kisspeptin protein expression was inhibited in the presence of inhibin A in mHypoA-55 cells (Fig. 4D).

Effect of inhibin A on activin-induced Kiss-1 gene expression in mHypoA-55 cells

Activin significantly increased Kiss-1 gene expression in mHypoA-55 ARC cells, but this effect was completely abolished in the presence of inhibin A. Activin increased Kiss-1 mRNA expression up to 2.23 ± 0.032-fold, but it was significantly reduced to 0.46 ± 0.11-fold by the combined treatment with activin and inhibin A (Fig. 5).

Effect of follistatin and inhibin A on kisspeptin- or GnRH-induced Kiss-1 gene expression in mHypoA-55 cells

Next, we tested the effect of follistatin and inhibin A on kisspeptin- or GnRH-induced Kiss-1 gene expression. Both kisspeptin and GnRH significantly increased the expression of Kiss-1 mRNA in mHypoA-55 ARC cells, up to 1.34 ± 0.10-fold by kisspeptin and up to 2.62 ± 0.48-fold by GnRH compared with untreated cells. Significant increases in Kiss-1 mRNA induced by kisspeptin or GnRH were almost completely abolished in the presence of follistatin and were reduced to the control level (1.14 ± 0.32-fold with kisspeptin and 1.18 ± 0.32-fold with GnRH) (Fig. 6A). Similarly, the kisspeptin-increased Kiss-1 gene expression in these cells was almost completely inhibited in the presence of inhibin A, which reduced it from 1.78 ± 0.23-fold to 1.10 ± 0.23-fold. GnRH-induced induction of Kiss-1 gene expression (3.33 ± 0.13-fold) was significantly inhibited in the presence of inhibin A (2.29 ± 0.2-fold) (Fig. 6B).

Effect of activin, follistatin, and inhibin A on Kiss-1 gene expression in the mHypoA-

50 AVPV cell model

Activin, follistatin, and inhibin A affected Kiss-1 gene expression in the mHypoA-55 ARC hypothalamic cell model. We next examined the effect of these peptides on mHypoA-50, a Kiss-1–expressing AVPV cell model. In contrast with the phenomenon observed in the mHypoA-55 ARC cell model, activin did not increase Kiss-1 gene expression in mHypoA-50 cells (Fig. 7A). Both follistatin and inhibin A modulated Kiss-1 gene expression in mHypoA-50 cells (Fig. 7B and C).

Expression of inhibin α, inhibin βA, inhibin βB, and follistatin in both mHypoA-55 and mHypoA-50 cells

RT-PCR analysis using specific primers for inhibin α, inhibin βA, and inhibin βB subunits demonstrated that the mRNAs of all three subunits were detected in tissues from the rat cerebral cortex, pituitary gland, and ovary. Furthermore, hypothalamic ARC and AVPV model mHypoA-55 and mHypoA-50 cells expressed all of these inhibin subunits (Fig. 8A). The follistatin gene was also detected in the cDNAs from the two hypothalamic cell models, rat brain cortex, and anterior pituitary tissues (Fig. 8A). Western blotting analysis using specific antibodies revealed that inhibin βA, βB, and follistatin proteins were expressed in both mHypoA-55 and mHypoA-55 cells; however, their expression was significantly higher in mHypoA-55 ARC cells than in mHypoA-50 AVPV cells. The level of inhibin α subunit was not significantly different in these cells (Fig. 8B and 8C).

Effect of sex steroids on inhibin subunit and follistatin gene expression in mHypoA-55 ARC cells

Finally, we examined whether inhibin subunit and follistatin genes expressed in mHypoA-55 ARC cells were influenced by the sex steroid E2. Cells were treated with E2 for 48 h and then mRNA levels for inhibin α, βA, and βB subunits, and follistatin were determined. E2 at 10 nM failed to increase the expression of these three inhibin subunits and follistatin. However, a higher concentration of E2 did induce changes in the expression of some of these peptides in these cells. Inhibin α subunit gene expression was significantly increased up to 3.00 ± 0.37-fold by 100 nM E2 compared with non-treated cells (Fig. 9A). However, inhibin βA and βB subunits were not significantly increased when cells were treated with 100 nM E2 (Fig. 9B and C). Follistatin gene expression in mHypoA-55 cells was significantly upregulated to 2.89 ± 1.32-fold by E2 compared with non-treated cells (Fig. 9D).

Discussion

Activin, inhibin, and follistatin proteins were originally reported to be produced in the gonads and believed to influence pituitary gonadotropin synthesis and secretion, especially FSH, but not LH [29]. In this study, we used two hypothalamic cell models, mHypoA-55 and mHypoA-50 Kiss-1–expressing cells. mHypoA-55 cells have characteristics of KNDy neurons in the ARC region of the hypothalamus, and the Kiss-1 gene in these cells was repressed by E2 under certain conditions. In contrast, E2 upregulated the Kiss-1 gene in HypoA-50 Kiss-1–expressing cells that originated from the AVPV region of the hypothalamus. These two cells are used as Kiss-1–expressing cell models for the study of E2-induced negative and positive feedback control [25]. Using these cells, we revealed that activin, inhibin, and follistatin might be involved in the regulation of Kiss-1 gene expression in the ARC region of the hypothalamus. Using the ARC Kiss-1–expressing cell model mHypoA-55, we found that (1) activin stimulation resulted in the expression of Kiss-1 mRNA; (2) follistatin reduced Kiss-1 gene expression and abolished activin's effect on the Kiss-1 gene; (3) inhibin A, but not inhibin B, reduced Kiss-1 gene expression and inhibin A prevented activin's effect on Kiss-1 expression; (4) both follistatin and inhibin A prevented kisspeptin- or GnRH-induced increases in Kiss-1 gene expression; and (5) inhibin α subunit and follistatin gene expression was upregulated by the sex steroid E2. Using the Kiss-1–expressing AVPV cell model mHypoA-50, we also found that activin did not have a stimulatory effect, and both follistatin and inhibin A did not have an inhibitory effect on Kiss-1 gene expression. Interestingly, expression levels of inhibin βA and βB subunits and follistatin were distinct between these two hypothalamic cell models.

Previous studies demonstrated that activin and inhibin subunits were distributed

in the hypothalamus in rodent brain and that activin βA subunit immunoreactivity in the hypothalamus was located in close proximity to the GnRH neurons [30, 31]. Activin βA and βB subunits as well as follistatin were also distributed in human brain including the hypothalamus [23, 24]. Furthermore, a previous in vivo study demonstrated that intracerebroventricular infusion of activin significantly increased the serum level of LH, but not FSH, in adult male rats, suggesting the interaction between activin and GnRH neuronal systems in the hypothalamus [30]. Our study using the hypothalamic Kiss-1–expressing model that originated from the ARC region of the hypothalamus, mHypoA-55, clearly showed that activin could stimulate Kiss-1 gene expression, indicating that activin may act at the hypothalamus and activate the HPG axis by modulating Kiss-1 gene expression in the ARC region of the hypothalamus in vivo. Interestingly, follistatin and inhibin A, both of which are known to act as antagonists for activin in the synthesis and secretion of FSH from pituitary cells [15, 32], reduced basal expression of Kiss-1 in mHypoA-55 cells and also antagonized activin's effect on Kiss-1 expression in these hypothalamic neuronal cells. These observations suggest that these peptides participate in the regulation of kisspeptin expression within the ARC region of the hypothalamus, resulting in the regulation of the pulsatile secretion of GnRH. On the other hand, a different type of Kiss-1–expressing cell model, mHypoA-50, which originates from the AVPV region of the hypothalamus, did not respond to activin, follistatin, or inhibin A, implying that these peptides do not have a pivotal role in the regulation of the GnRH/LH surge.

Accumulating evidence implies that activins are broadly expressed in many tissues and organ systems and serve in multiple regulatory functions [33, 34]. Activin A is the most abundant and best-characterized member of the activin family and plays a

predominant role in activin signaling in the central nervous system. Activin A is induced and acts as a neuroprotective factor in various forms of acute brain injury or stroke [35, 36]. It also supports neuronal development and differentiation of neuronal stem cells or neuronal progenitor cells [37, 38]. Activin A is produced not only in neurons, but also in glial cells, and promotes oligodendrocyte differentiation and remyelination [39]. Activin signaling was also reported to be involved in fear conditioning and memory [40] and anxiety [41]. Considering previous reports about the functions of activin and current observations that hypothalamic Kiss-1–expressing cells also express activin, we could speculate that activin induced within the brain affects the HPG axis by increasing Kiss-1 gene expression. Because serum levels of activin are known to be extremely low, it is generally agreed that activin acts in an autocrine-paracrine manner only in limited areas. Both activin and follistatin were originally identified as gonadal peptides that have the ability to stimulate or inhibit FSH secretion from the pituitary [13, 42]; these peptides are expressed within the pituitary and work to regulate FSH in an autocrine/paracrine manner. Furthermore, activin and follistatin expressed in the pituitary gland are upregulated by GnRH [43, 44]. On the other hand, it is believed that circulating inhibins that are produced in the gonads act at the pituitary and inhibit FSH release [28]. Therefore, we speculated that locally produced activin and/or follistatin within the hypothalamus participate in the regulation of the Kiss-1 gene.

Interestingly, inhibin A, but not inhibin B, prevented activin's effect on Kiss-1 gene expression. In humans, serum levels of inhibin A and B change differently during the menstrual cycle. The plasma concentration of inhibin B rises rapidly in the early follicular phase on the day after the intercycle FSH rise, then falls progressively during the remainder of the follicular phase. After the LH surge, there is a short-lived peak in

inhibin B concentration, but it then falls to low concentrations during the remainder of the luteal phase. In contrast, the inhibin A concentration is low in the early follicular phase, rises at ovulation, and is maximal during the midluteal phase [28]. Because inhibin B, but not inhibin A, significantly reduced the expression of Kiss-1 mRNA by itself in mHyoA-55 cells, we speculate that inhibin A, which is predominantly released from lutein cells in the ovary, exerts a negative feedback on the hypothalamus, resulting in the reduction of the pulsatile release of GnRH and subsequent decrease in gonadotropin secretion.

It is noteworthy that although both follistatin and inhibin A exert their inhibitory effects as activin antagonists, they also prevent the increase in Kiss-1 gene expression induced by stimulants other than activin in mHypoA-55 ARC model cells. We have previously demonstrated that both kisspeptin and GnRH stimulate Kiss-1 gene expression in primary cultures of neuronal cells from fetal rat brain [45]. In mHypoA-55 cells, both kisspeptin and GnRH increase Kiss-1 gene expression, but this effect was abolished or significantly prevented in the presence of follistatin or inhibin B. Activins signal through heteromeric complexes of type II (ActRIIA and ActRIIB) and type I receptors (ActRIB, ActRIB, and ActRIC). Type II receptors bind activin and recruit type I receptors, and then transduce their signals by phosphorylating SMAD protein [46]. Follistatin binds directly and tightly with activin and hinders activin's binding to its receptor [20], whereas inhibin counteracts activin binding to its receptor by binding betaglycan, which has high affinity for ActRII [47]. On the other hand, both Kiss1R and the GnRH receptor (GnRHR) couple with Gq/11 and induce the formation of inositol triphosphate and diacylglycerol through phospholipase C–dependent mechanisms, and both activate extracellular signal-regulated kinase signaling pathways in a protein kinase C–dependent manner [48-50]. Follistatin and inhibin act at the level of the activin receptor and they may not interact with Kiss1R

and GnRHR. Considering observations that both follistatin and inhibin B significantly reduced the basal expression of Kiss-1 mRNA by themselves, these peptides might directly affect gene expression without activin or activin receptors.

Previous studies have demonstrated that inhibin subunits, which compose activin and inhibin, were broadly or ubiquitously expressed in the hypothalamic brain region in rodents and humans [30, 31]. However, considering the expression pattern of inhibin βA, βB, and follistatin genes in mHypoA-55 and mHypoA-50 cells, it is plausible that the expression pattern of inhibin subunits and follistatin was distinct in different parts of the hypothalamic nucleus. Because basal expression of inhibin βA, βB, and follistatin genes was higher in ARC-derived mHypoA-55 cells than in AVPV-derived mHypoA-50 cells, activin/follistatin/inhibins may be present at higher levels in the ARC region and participate in the regulation of the HPG axis. In this study, we demonstrated that inhibin subunit and follistatin genes were present in Kiss-1–expressing cell models derived from both ARC and AVPV regions of the hypothalamus; however, details are still unknown as to whether these genes are coexpressed in vivo, and whether autocrine regulation of the Kiss-1 gene occurs in the ARC region. Furthermore, it still not known how the expression of activin, inhibins, and follistatin changes during reproductive cycles in vivo. It is also still unclear how these peptides are regulated within the hypothalamus. In this study, we found that E2 induced inhibin α subunit and follistatin gene expression in mHypoA-55 ARC cells, suggesting that activin's effects or the inhibitory effect of inhibin A/follistatin on Kiss-1 expression is influenced by E2 within the ARC region of the hypothalamus. Further in vivo studies are needed to elucidate the details of local activin/inhibin/follistatin expression and actions in the hypothalamus.

In this study using the hypothalamic Kiss-1–expressing cell model from the ARC

region, mHypoA-55, we found that activin could increase the expression of Kiss-1, whereas both follistatin and inhibin B could decrease Kiss-1 expression. The activin/inhibin/follistatin system might work not only in pituitary FSH regulation, but also at the level of the hypothalamus, and maintain the HPG axis.

Disclosure statement

The authors have nothing to disclose.

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Figure Legends

Figure 1

Effect of activin on Kiss-1 mRNA expression in mHypoA-55 ARC cell models. (A) mHypoA-55 cells were stimulated with 1 ng/ml and 10 ng/ml activin A for 24 h. (B) mHypoA-55 cells were stimulated with 10 ng/ml activin A for the indicated times. mRNA was then extracted and reverse transcribed, and Kiss-1 mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold induction over unstimulated cells and presented as mean ± SEM values of three independent experiments, each performed with duplicate samples. *$P < 0.05$ vs. control. Statistical significance was determined by one-way ANOVA with Bonferroni's post hoc test. (C) mHypoA-55 cells were stimulated with 10 ng/ml activin A for 24 h and then cell lysates (30 μg protein) were analyzed by SDS-PAGE followed by immunoblotting and incubation with antibodies against kisspeptin. β-Actin was detected as an internal control. The bands were visualized using an HRP-conjugated secondary antibody.

Figure 2

Effect of follistatin on Kiss-1 mRNA expression in the mHypoA-55 ARC cell model. (A) mHypoA-55 cells were stimulated with 1 ng/ml and 10 ng/ml follistatin for 24 h. (B) mHypoA-55 cells were stimulated with 10 ng/ml follistatin for the indicated times. mRNA was then extracted and reverse transcribed, and Kiss-1 mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold induction over unstimulated cells and presented as mean ± SEM values of three independent experiments, each performed with duplicate samples. *$P < 0.05$ vs. control. Statistical significance was

determined by one-way ANOVA with Bonferroni's post hoc test. (C) mHypoA-55 cells were stimulated with 10 ng/ml follistatin for 12 h and then cell lysates (30 μg protein) were analyzed by SDS-PAGE followed by immunoblotting and incubation with antibodies against kisspeptin. β-Actin was detected as an internal control. The bands were visualized using an HRP-conjugated secondary antibody.

Figure 3

Effect of follistatin on activin-induced expression of Kiss-1 mRNA in mHypoA-55 ARC cells. mHypoA-55 cells were stimulated with 10 ng/ml activin A in the presence or absence of 10 ng/ml follistatin for 24 h, after which mRNA was extracted and reverse transcribed. Kiss-1 mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold induction over unstimulated cells and presented as mean ± SEM values of three independent experiments, each performed with duplicate samples. $*P < 0.05$ vs. control. Statistical significance was determined by one-way ANOVA with Bonferroni's post hoc test.

Figure 4

Effect of inhibins on Kiss-1 mRNA expression in mHypoA-55 ARC cells. mHypoA-55 cells were stimulated with 1 ng/ml and 10 ng/ml inhibin A (A) or inhibin B (B) for 24 h, after which mRNA was extracted and reverse transcribed. (C) mHypoA-55 cells were stimulated with 10 ng/ml inhibin A for the indicated times. mRNA was then extracted and reverse transcribed and Kiss-1 mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold induction over unstimulated cells and presented as mean ± SEM values of three independent experiments, each performed with duplicate

samples. *P < 0.05 vs. control. Statistical significance was determined by one-way ANOVA with Bonferroni's post hoc test. (D) mHypoA-55 cells were stimulated with 10 ng/ml inhibin A for 12 h and then cell lysates (30 μg protein) were analyzed by SDS-PAGE followed by immunoblotting and incubation with antibodies against kisspeptin. β-Actin was detected as an internal control. The bands were visualized using an HRP-conjugated secondary antibody.

Figure 5

Effect of inhibin A on activin-induced expression of the Kiss-1 gene in mHypoA-55 ARC cells.

mHypoA-55 cells were stimulated with 10 ng/ml activin A in the presence or absence of 10 ng/ml inhibin A for 24 h, after which mRNA was extracted and reverse transcribed. Kiss-1 mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold induction over unstimulated cells and presented as mean ± SEM values of three independent experiments, each performed with duplicate samples. **P < 0.01, *P < 0.05 vs. control. Statistical significance was determined by one-way ANOVA with Bonferroni's post hoc test.

Figure 6

Effect of follistatin and inhibin A on kisspeptin- or GnRH-induced expression of the Kiss-1 gene in mHypoA-55 ARC cells. mHypoA-55 cells were stimulated with 10 nM kisspeptin (KP10) or GnRH in the presence or absence of 10 ng/ml follistatin (A) and inhibin A (B) for 24 h, after which mRNA was extracted and reverse transcribed. Kiss-1 mRNA levels were measured by quantitative real-time PCR. Results are expressed as the

fold induction over unstimulated cells and presented as mean ± SEM values of three independent experiments, each performed with duplicate samples. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control. Differences between KP10 and KP10 + follistatin, between GnRH and GnRH + follistatin, between KP10 and KP10 + inhibin A, and between GnRH and GnRH + inhibin A were statistically significant. Statistical significance was determined by one-way ANOVA with Bonferroni's post hoc test.

Figure 7

Effect of activin, follistatin, and inhibin A on Kiss-1 gene expression in mHypoA-50 AVPV cells. mHypoA-50 cells were stimulated with the indicated concentrations of activin A (A), follistatin (B), or inhibin A (C) in for 24 h. mRNA was then extracted and reverse transcribed and Kiss-1 mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold induction over unstimulated cells and presented as mean ± SEM values of three independent experiments, each performed with duplicate samples.

Figure 8

Expression of inhibin subunits and follistatin in mHypoA50 and mHypoA-55 cells. (A) Total RNA was prepared and RT-PCR was carried out for 40 cycles using primers specific for inhibin α, inhibin βB, and inhibin βA subunits, and follistatin. PCR products were resolved in a 1.5% agarose gel and visualized with ethidium bromide staining. (B) Cell lysates (30 μg protein) from mHypoA50 and mHypoA-55 cells were analyzed by SDS-PAGE followed by immunoblotting and incubation with antibodies against inhibin α, inhibin βB, and inhibin βA subunits and follistatin. The bands were visualized using

HRP-conjugated secondary antibody. (C) Scanning densitometry of visualized bands using NIH ImageJ software was performed to determine differences in protein expression, normalized to that of β-actin, between mHypoA-50 and mHypoA-55 cells. $^{**}P < 0.01$ vs. mHypoA-50. Statistical significance was determined by Student's *t* test.

Figure 9

Effect of E2 on inhibin subunits and follistatin expression in mHypoA-55 cells. mHypoA-55 cells were stimulated with 10 nM and 100 nM E2 for 48 h, after which mRNA was extracted and reverse transcribed. Inhibin α (A), inhibin βA (B), inhibin βB subunit (C) and follistatin (D) mRNA levels were measured by quantitative real-time PCR. Results are expressed as the fold induction over unstimulated cells and presented as mean ± SEM values of three independent experiments, each performed with duplicate samples. $^{**}P < 0.01$ vs. control. Statistical significance was determined by one-way ANOVA with Bonferroni's post hoc test. n.s.; difference was not significantly different.